Cell Adhesion Molecule Expression on Vascular Endothelial Cells in Human Gastrointestinal Carcionomas

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博士論文

Cell Adhesion Molecule Expression on Vascular Endothelial Cells in Human Gastrointestinal Carcinomas

胃大腸癌組織における腫瘍血管に発現する細胞接着分子の意義

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Backgrounds: The cell adhesion between vascular endothelial cells and inflammatory cells is an important process for the immuno-inflammatory reaction. In inflammatory diseases, a correlation has been demonstrated between the expression of the cell adhesion molecules in vascular endothelial cells and the degree of inflammatory infiltrates in situ. In cancer tissue, infiltration of inflammatory cells has been suggested to be a host resistance. To clarify this mechanism, we applied the above-mentioned theory to human gastrointestinal carcinoma.

Experimental design: We analyzed correlation between the immunohistochemical localization of the cell adhesion molecules (E-selectin, P-selectin, ICAM-1) on tumor vessels and distribution of inflammatory infiltrates in gastrointestinal carcinoma.

Results: Venules in the invasion front of colorectal and intestinal-type gastric carcinomas consistently expressed E-and P-selectins and ICAM-1 ("immunologically activated venules"). Contrasted with this, most of blood vessels within the tumor lacked immunoreactivity for all these adhesion molecules ("immunologically inactive vessels"). Granulocytes, lymphocytes and macrophages were more densely distributed in the invasion front, while a few of them were present within the tumor. Their numbers in the invasion front were significantly lower in a group of colon carcinoma with hepatic metastasis.

Conclusion: The adhesion molecules expressed by endothelial cells may regulate the inflammatory infiltrates in cancer tissue. The behavior of carcinoma is influenced by these cell infiltrates. We emphasize the importance of the comparative study between inflammatory diseases and the host reaction to cancer in the study of tumor biology.

Additional Key Words: selectins, ICAM-1, liver metastasis, tumor vessels, immunohistochemistry.

INTRODUCTION

In 1863 Virchow first identified inflammatory infiltrate in and at the edge of cancer tissue. These infiltrates have been suggested to be the manifestations of the host reaction, associated with a better prognosis in carcinomas of the breast (1, 2), stomach (3) and colon (4-7). Lymphocytes isolated from human colon cancer were cytotoxic *in vitro* to the cancer cells from the same patient (8). On the other hand, lymphoreticular infiltration in malignant melanoma has been correlated with a poor survival rate (9). These conflicting results suggest pleiotypic functions of these inflammatory infiltration to the cancer cells in general.

In colorectal carcinoma, inflammatory infiltrates are particularly dense around the periphery of the tumor, i.e., in the invasion front (10, 11). However, the exact mechanism of the host side responsible for this phenomenon has not been elucidated.

Recently, it has been demonstrated that leukocyte trafficking and recruitment to the inflammatory sites are mediated by the cell adhesion molecules on endothelial cells (12-15). E-selectin (ELAM-1)(16) and P-selectin (CD62, GMP140)(17, 18) contain the N-terminal lectin domain that mediates the adhesion by binding to the carbohydrate ligands on leukocytes. These two selectins are expressed on activated endothelial cells (E-and P-selectins) and platelets (P-selectin). They are involved in the recruitment of granulocytes, memory T cells, cells of a monocyte/macrophage lineage and natural killer cells to the sites of inflammation (19-22). Intercellular adhesion molecule-1 (ICAM-1), a member of the immunoglobulin superfamily, is also expressed on endothelial cells and inflammatory cells, which binds to the leukocyte function

associated antigen-1 (LFA-1) expressed on leukocyte cell surface (23).

We have already demonstrated a correlation between the expression of the cell adhesion molecules in activated venules and the degree of inflammatory infiltrates *in situ* in inflammatory bowel disease (24, 25). In cancer tissue, most investigations on the tumor vessels have focused their attention to the mechanism of neovascularization and the relation between angiogenesis and tumor growth (26-28). Blood vessels have been regarded as "ducts" to supply the nutrition to cancer tissue.

In the present study, we investigated the correlation between immunolocalization of the cell adhesion molecules in blood vessels and distribution of inflammatory infiltrates bearing their counter-receptors. E- and P-selectins were mainly analyzed since they were the best marker of immunologically activated venules (24). Vascular cell adhesion molecule-1 (VCAM-1) was not studied because it was not expressed in activated venules either in inflammatory bowel disease (24) or in cancer tissue (our preliminary data). We will describe that there are two types of blood vessels in the tumor tissue; i.e., blood vessels in the host side of invasion front as a immuno-inflammatory reaction and blood vessels in the tumor cell nests which function as nutritional vessels.

EXPERIMENTAL DESIGN

We used surgically resected human specimens of colorectal (20 cases) and gastric carcinomas (24 cases) obtained at Tohoku University Hospital and Tohoku Rosai Hospital. Immediately after surgical resection, small slices of specimens, 5x5x2mm in size, were fixed in periodate-lysine-4%paraformaldehyde (4%PLP) (29) for 6-8h. After washing in phosphate-buffered saline (PBS) containing 10%, 15% and 20% sucrose, the specimens were embedded in O.C.T.compound (Miles, Elk hart, USA) and rapidly frozen in acetone-dry ice. For the control, normal-appearing colorectal and gastric tissues were obtained remote from the carcinoma. Gastric carcinomas were histopathologically classified into intestinal (13 cases) and diffuse (9 cases) types (30). Two cases were mixed type. Colorectal carcinomas were either well or moderately differentiated adenocarcinoma (31). All cases showed invasion into or beyond the muscularis propria.

Serial frozen sections were stained with monoclonal antibodies as listed in Table 1. Staining was performed by the indirect immunoperoxidase method. Immunoelectron microscopic study was performed for E-and P-selectins as described previously (32) in two cases of colon carcinoma and one case of diffuse-type gastric carcinoma.

The numbers of inflammatory infiltrates ($CD3^+$ cells, $CD4^+$ cells, granulocytes and $CD68^+$ macrophages) were calculated in tissue specimens of colorectal carcinoma at the invasion front as described in the method section, and they were compared between groups with and without hepatic metastasis by the paired t-test.

RESULTS AND DISCUSSION

IDENTIFICATION AND CLASSIFICATION OF TUMOR VESSELS

Vascular structure was confirmed by immunoreactivity for laminin and CD31. Tumor vessels were classified into three groups based on the immunoreactive pattern for the cell adhesion molecules and their location; i.e., a) blood vessels in the stroma in the close proximity of tumor cell nests described as "vessels in the tumor", b) blood vessels in the intervening stroma and c) blood vessels in the host side of tumor invasion front described as "vessels in the invasion front" (cf. Fig. 6). The intervening stroma is defined as connective tissue between the clusters of tumor cell nests.

IMMUNOHISTOCHEMICAL STUDY

1. COLORECTAL CARCINOMA

In colorectal carcinoma, blood vessels increased in the tumor, intervening stroma and invasion front (Fig. 1a). E-and P-selectins and ICAM-1 were consistently positive in all cases in the endothelial cells of venules in the invasion front (Figs. 1b, 2a, 2c). The diameter of these venules ranged from 10 to 100μm. CD41 staining revealed immunoreactive platelets in the lumen of venules and no staining was observed in endothelial cells (data not shown, see Ref. 24). Their phenotypical features were the same as "immunologically activated venules" in inflammatory lesions (24). However, none of capillaries and venules in the tumor showed immunoreactivities for the cell adhesion molecules (Figs. 1b, 2b). We have defined these vessels as "immunologically inactive vessels" (see the discussion section for details). In the intervening stroma, some of the venules were positive for E-and P-selectins and ICAM-1 (data not shown). In one case, venules positive for E-

and P-selectins appeared in the intervening stroma as the same degree as in the invasion front.

Distribution patterns of inflammatory infiltrates are shown in Figs 1c, 1d, 3. Granulocytes (revealed by the endogenous peroxidase activity), $CD3^+$ and $CD4^+$ cells, and macrophages (revealed by CD68) were all distributed more densely around the invasion front. Contrasted with this, these inflammatory infiltrates were fewer in the tumor and in the intervening stroma. The area densely populated by inflammatory infiltrates corresponded to the area where venules expressed the cell adhesion molecules. This relationship was confirmed in 15 of 20 cases for $CD3^+$ and $CD4^+$ cells, in 13 of 20 cases for granulocytes and in 39 of 63 cases for macrophages. Other cases showed inflammatory infiltrates also in the intervening stroma and in the tumor nearly at the same degree as in the invasion front.

Staining with frozen sections (Fig. 3a) revealed that most of sialyl $Lewis^X$-positive cells were inflammatory infiltrates (granulocytes, macrophages and some lymphocytes) and that carcinoma cells were occasionally positive.

These results are schematically summarized in Fig. 6.

2. GASTRIC CARCINOMA OF INTESTINAL TYPE

The results were essentially the same as those in colorectal carcinoma. In most cases, venules expressing the cell adhesion molecules were detected mainly in the invasion front with dense inflammatory infiltrates in the same area. In two cases with abundant stroma, vessels expressing the cell adhesion molecules were more often found in the intervening stroma, where cell infiltration were observed at the same degree as in the invasion front.

3. GASTRIC CARCINOMA OF DIFFUSE TYPE

The vascular pattern was totally different from that of colorectal carcinoma. This group lacked the vascular reaction in the invasion front, which was consistently observed in colorectal carcinoma. There was a little phenotypic difference between vessels in the tumor and those in the intervening stroma. Immunohistochemically, some venules or capillaries expressed E- and P-selectins and ICAM-1 in the whole area of cancer (Fig 4). The numbers of inflammatory infiltrates varied with cases, but no cases showed accumulation of them in the invasion front.

4. CHANGES AT THE ULCER BASE.

Carcinoma tissues were frequently associated with ulceration. Ulcer bases in carcinoma abounded with venules expressing the cell adhesion molecules and with inflammatory infiltrates (data not shown). These were apparently reactive to inflammatory changes at the ulcer bases.

5. NORMAL TISSUE

Endothelial cells of venules were sporadically positive for E- and P-selectins as stated in our previous paper (24,25). Compared with total vessels identified by laminin and CD31, ICAM-1 was consistently expressed in most of capillaries and venules (24). Small numbers of granulocytes, lymphocytes and macrophages were observed in the lamina propria.

IMMUNOELECTRON MICROSCOPY

Immunoreactivity for E- and P-selectins was continuously localized along the luminal plasma membrane of endothelial cells of venules in the invasion front (Figs. 5a,b). This localization is consistent with their character as transmembrane receptor

proteins. This also excluded the possibility that immunoreactivity for P-selectin represents platelets aggregated on endothelial cells. Occasionally, E- and P-selectins were observed in round granules (25,33) and in the Weibel-Palade bodies in endothelial cells, respectively (data not shown). We did not observe remarkable expression of E-selectin in rough endoplasmic reticulum or frequent occurrence of exocytosis of E-selectin into the vascular lumen as observed in inflammatory bowel disease (25). This suggested that endothelial activation in the present study was not so remarkable as in active inflammatory area in inflammatory bowel disease.

INFLAMMATORY INFILTRATES AND LIVER METASTASIS

The numbers of $CD3^+$ and $CD4^+$ cells and $CD68^+$ macrophages in the invasion front were larger in a group of colorectal carcinoma without hepatic metastasis than those with hepatic metastasis ($p<0.05$)(Figs 7 and 8). The number of granulocytes was also larger in cases without metastases, but not statistically significant (data not shown).

DISCUSSION

This is the first paper which describes the expression of cell adhesion molecules by vascular endothelial cells in cancer tissue from the viewpoint of host reaction to cancer cell invasion. We demonstrated the following points; a) there was a correlation between the expression of cell adhesion molecules on venules and the distribution of inflammatory infiltrates, b) phenotypical heterogeneity was observed between blood vessels in the invasion front and those in the tumor, and c) the numbers of inflammatory

infiltrates in the invasion front were reversely correlated with the presence of hepatic metastasis in colorectal carcinoma.

It is now well documented that the cell adhesion molecules play important roles in the tumor invasion and metastasis (34). However, we considered the significance of the expression of cell adhesion molecules from a different standpoint. We analyzed the functional aspects of the cell adhesion molecules in the endothelial cells by the analogy of those in active inflammatory diseases.

In colorectal and intestinal-type gastric carcinomas, venules expressing the cell adhesion molecules occurred particularly in the invasion front, where inflammatory infiltrates were accumulated. Contrasted with this, blood vessels in the tumor were negative for these adhesion molecules, where less inflammatory infiltrates were present. These results suggest that infiltration of inflammatory cells in cancer tissue was regulated by the cell adhesion molecules expressed by the vascular endothelial cells. We have already defined these vessels as "immunologically activated vessels" in inflammatory bowel disease (24).

In colorectal carcinoma, increased infiltration of eosinophils (7), macrophages (35) and lymphocytes (36) are associated with a better survival rate. Furthermore, the inflammatory infiltration in the invasion front is a more significant indicator of prognosis than that observed in the central part of carcinoma (10, 11, 36). Our present data also revealed more pronounced cell infiltration in the group without hepatic metastasis. Therefore, we considered that inflammatory infiltrates (=immuno-competent cells) in the invasion front act as a defensive mechanism against the carcinoma cell invasion.

We noticed that vessels in the tumor were negative for the cell adhesion molecules. Lack of E-selectin was already observed in the vessels within basal cell carcinoma of the human skin (37). In experimental model, the interaction between leukocytes and vascular endothelial cells was diminished in tumor microvessels (38). TNF-α and IL-1 stimulate the expression of E-and P-selectins and ICAM-1 (23). Therefore, this lack of the cell adhesion molecules may be resulted from a lack of stimulation by these cytokines in the tumor. However, TNF-α mRNA was detected within the tumor in colon carcinoma (39, 40). Therefore, we speculate that endothelial cells in the tumor lack a capacity to express the cell adhesion molecules influenced by certain microenvironments in the tumor. We named these vessels as "immunologically inactive vessels". They probably function solely as "nutritional vessels" for the tumor cells.

In diffuse-type gastric carcinoma, the expression pattern of the cell adhesion molecules was totally different from those of colorectal and intestinal-type gastric carcinomas. Another phenotypical difference of microvessels in diffuse-type gastric carcinoma was already reported on von Willebrand factor, one of representative markers of endothelial cells (33). There may be a correlation between this specific vascular phenotype and the specific biological behaviors of diffuse-type cancer; i.e., diffuse infiltration of cancer cells, lack of hepatic metastasis or high incidence of the peritoneal dissemination.

Several carbohydrate antigens, previously defined as cancer associated antigens, are ligands for selectins including sialyl Lewis^{X} and sialyl Lewis^{a} (CA19-9)(19, 41-43). Based on the assumption that carbohydrate moiety mediate the adhesion of

cancer cells to endothelial cells, many reports described the correlation between metastasis and the expression of these carbohydrate antigens in cancer cells (34, 44). However, the present study revealed that the major cells expressing sialyl Lewis^X antigen are stromal cells, not cancer cells. This difference may be resulted from the difference of methodology; we used frozen sections where glycolipids were well preserved (45). Antigenicity for carbohydrate antigens in host cells may be reduced considerably in paraffin-embedded sections. Therefor, we speculate that the major role of the cell adhesion molecules expressed in human cancer tissue is the immuno-inflammatory interaction.

In conclusion, we have demonstrated the following findings; a) the invasion front is an area of immuno-inflammatory reaction to cancer cell invasion, and b) area within the tumor is an immunologically inactive area where immuno-competent cells have a limited access. Our results may have important implications for the cancer therapy; e.g., to modulate phenotypical characters of blood vessels in the tumor to enhance the access of immuno-competent cells. Much detailed characters may be disclosed by the observation of immuno-inflammatory reaction. Therefore, we emphasize the importance of the comparative study between the host reaction to cancer cell invasion and inflammatory diseases.

METHODS

IMMUNOHISTOCHEMISTRY

Frozen sections, 6μm in thickness, were cut with a cryostat and mounted on ovalbumin-coated glass slides. After immersing in non-immunized sheep serum, the indirect immunoperoxidase method was applied. The first antibodies (listed in Table. 1) were applied to the sections for 24 hours. Endogenous peroxidase activity was blocked by treating the slides with 0.3% hydrogen peroxidase in methanol for 12minutes. The second antibodies were sheep peroxdase-conjugated $F(ab')_2$ fragment of anti-mouse and anti-rat immunoglobulins (Amersham, United Kingdom). These were diluted at 1:150 by PBS containing 5% human serum and applied over-night. The enzymatic reaction was performed in 0.3% 3'-3 diaminobenzidine tetrahydrochloride (DAB) solution containing 0.065% sodium azide.

IMMUNOELECTRON MICROSCOPY

The staining procedure was the same as in light microscopic immunohistochemistry except omitting the immersion of specimens in methanol with hydrogen peroxide. After DAB reaction, the specimens were fixed 1% osmium tetroxide for 1 hour, dehydrated ethanol and embedded in Epon. Ultrathin sections were stained with lead citrate for 2min and observed with a JEM-100B electron microscope.

CELL COUNTING

The numbers of $CD3^+$ and $CD4^+$ cells and granulocytes were calculated at the site of invasion front using frozen sections of 20 cases of colorectal carcinoma (8 cases with hepatic metastasis and

12 cases without hepatic metastasis). Three representative fields were chosen where average numbers of inflammatory infiltrates were present in each case. Immunoreactive cells bearing nucleus were counted in the microgrids using ×400 field. The grid covered an area of 0.065mm^2. CD68$^+$ macrophages were counted by the same method using paraffin-embedded blocks in 63 colorecatal carcinomas (40 cases without hepatic metastasis and 23 cases with hepatic metastasis).

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Legends for Figures

Figure. 1. A characteristic immunostaining pattern of colorectal carcinoma. Vascular structure is revealed by immunostaining for laminin (a). E-selectin (b) is expressed exclusively in venules in the invasion front (lower half), not by blood vessels in the tumor (upper half). Granulocytes (c) revealed by the endogenous peroxidase activity and $CD3^+$ T lymphocytes (d) were distributed more densely in the invasion front than in the tumor. a-d) ×50. Scale bar = 200μm.

Figure. 2. Immunostaining for P-selectin (a,b) and ICAM-1 (c) in colorectal carcinoma. P-selectin is positive in venules in the invasion front (a), but totally negative in vessels in the tumor (b). This pattern is identical to that of E-selectin. ICAM-1 is also expressed in venules in the invasion front (arrows) as well as inflammatory infiltrates nearby. a-c) ×130. Scale bar = 100μm.

Figure. 3. Distribution patterns of inflammatory infiltrates in colorectal carcinoma. Cancer cells are located in the right half in all three figures. Sialy $Lewis^X$ + cells (a), $CD4^+$ T lymphocytes (b) and $CD68^+$ macrophage (c) are all distributed more densely around the invasion front. Note an abundance of sialyl $Lewis^{X+}$ cells there. a-c) ×130. Scale bar = 100μm.

Figure. 4. Immunostaing for the cell adhesion molecules in gastric carcinoma of diffuse type. Total vessels are revealed by laminin (a). Part of venules or capillaries express E- (b) and P- (c) selectins in the whole area of cancer. a-c) ×170. Scale bar = 50μm.

Figure 5. Immunoelectron microscopy of E- (a) and P- (b) selectins in colorectal carcinoma. Note continuos immunolabeling for both selectins along the luminal plasma membranes of endothelial cells. ×17,000. Scale bar = 0.5μm.

Figure 6. A schema of immunolocalization of cell adhesion molecules on tumor vessels and distribution of inflammatory infiltrates.

Figure 7. Cell counts of $CD3^+$ and $CD4^+$ T lymphocytes in the invasion front of colorectal carcinoma. The numbers of $CD3^+$ and $CD4^+$ cells are statistically larger in cases without hepatic metastasis than those with hepatic metastasis ($p<0.05$).

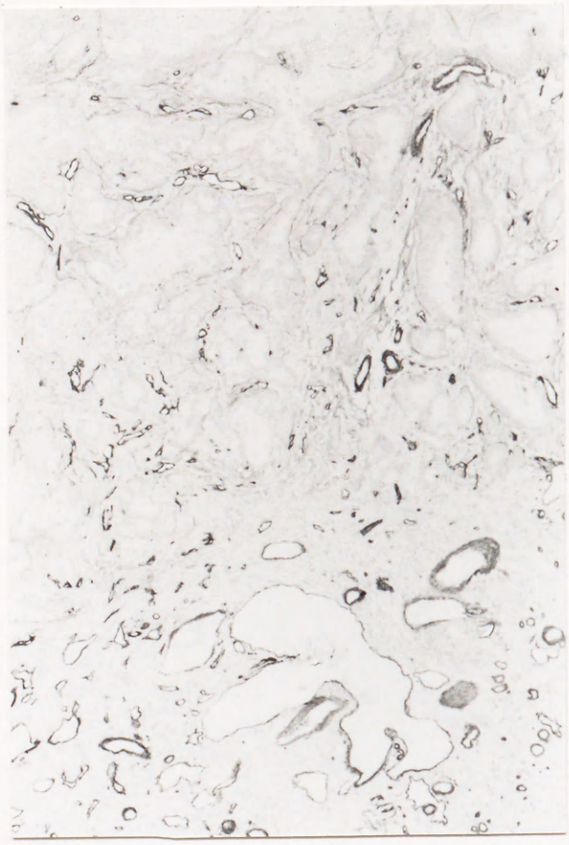
Figure 8. Distribution density of $CD68^+$ macrophages in the invasion front of colorectal carcinoma using paraffin-embedded blocks. The number of $CD68^+$ macrophages is larger in cases without hepatic metastasis than those with hepatic metastasis ($p<0.05$).

Table 1. List of the monoclonal antibodies used

Monoclonal antibodies	localization	Souces	Working dilution
E-selectin	activated endothelial cells	British biotechnology (BBA1)	1:500
P-selectin	activated endothelial cells and platelets	Takara (cloneWGA1) (Kyoto,Japan)	1:500
ICAM-1	endothelial cells activated macrophages etc	Immunotech (clone 84H10)	1:300
sialyl Lewis ^{x}	granulocytes macrophages, etc	Dr Fukushi (clone FH6)(46)	1:20
CD31	endothelial cells and some of lymphocytes	DAKO (clone JC/70A)	1:100
laminin	basement membrane	Immunotech (clone 4C12.8)	1:200
CD3	mature T lymphocytes	BECTON DIKINSON (clone SK7)	1:100
CD4	helper/inducer T lymphocytes	Nichirei (clone NU-TH/4)(Tokyo, Japan)	1:32
CD68	mono/macrophages	DAKO (clone PG-M1)	1:500

Fig 1

a



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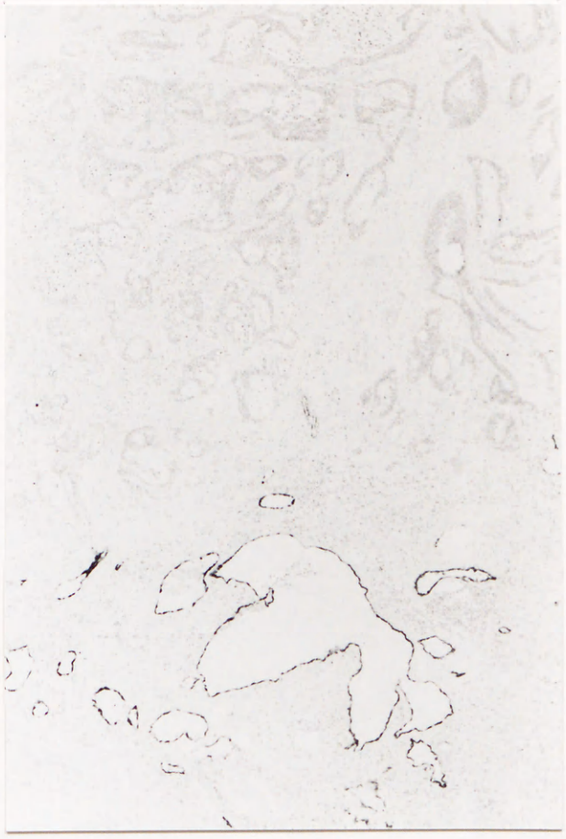

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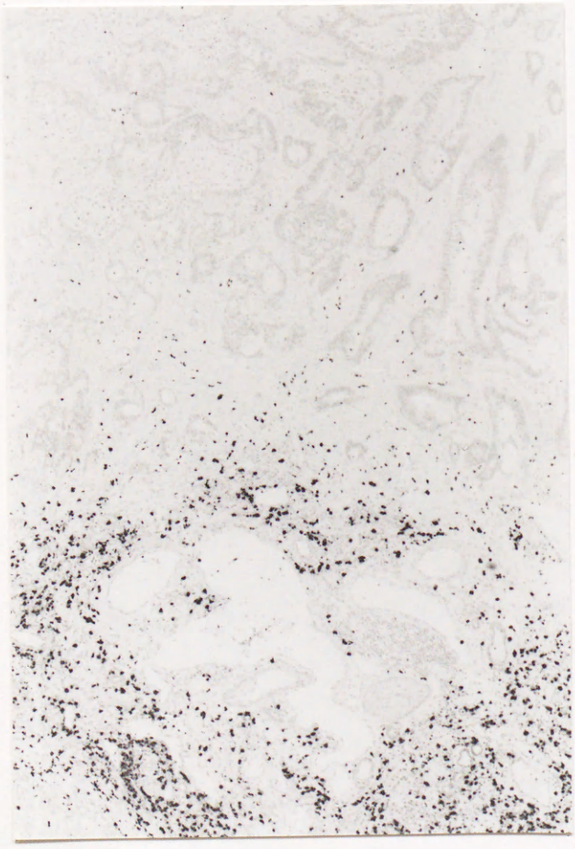


d

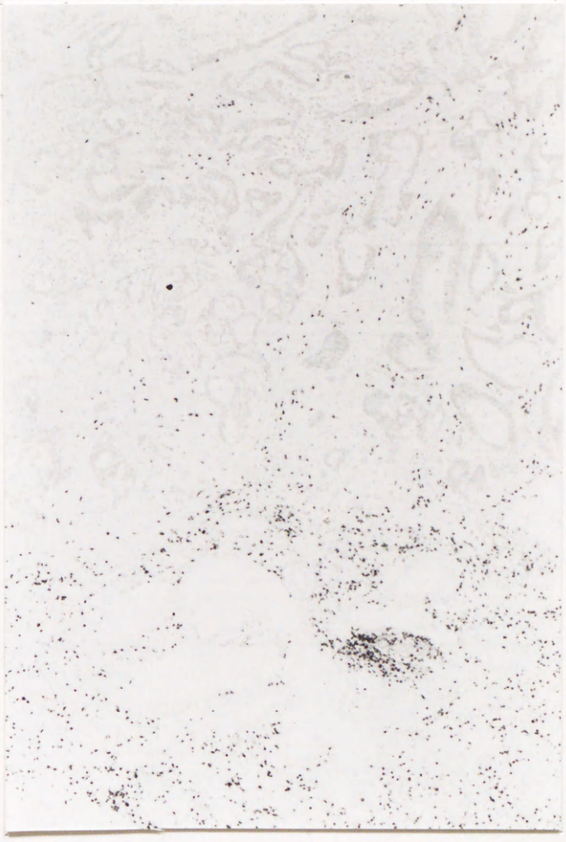
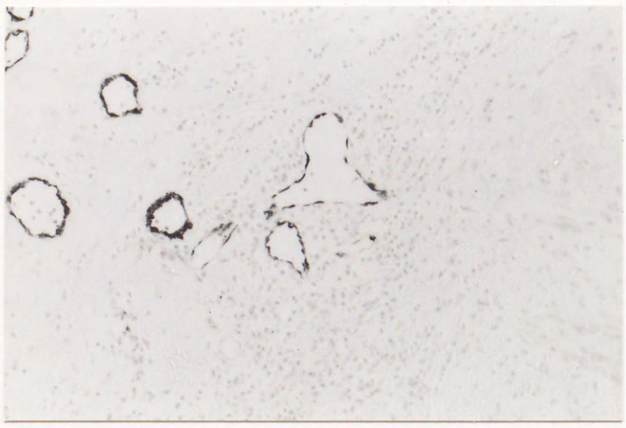


Fig 2

a



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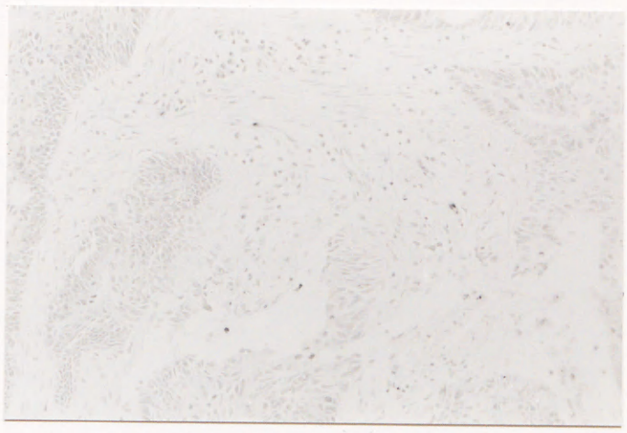
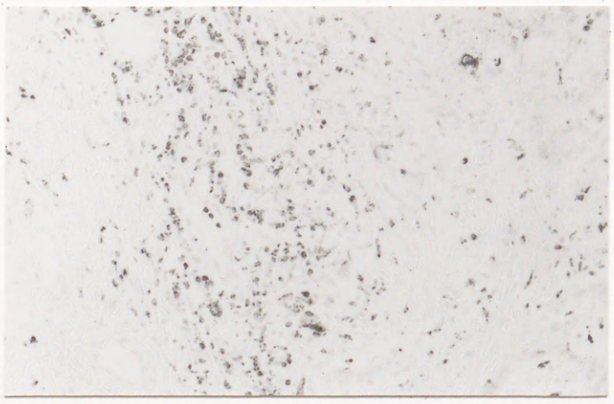


c

Fig 3

a



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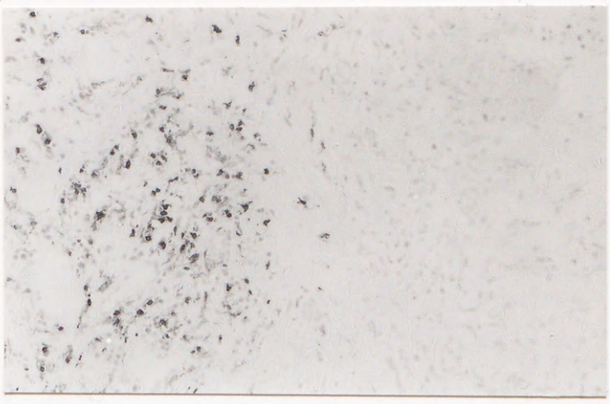

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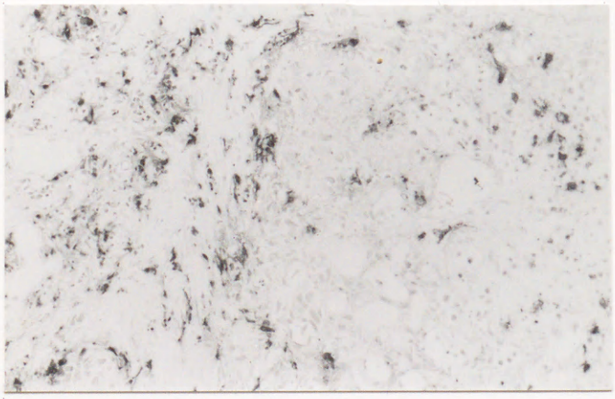
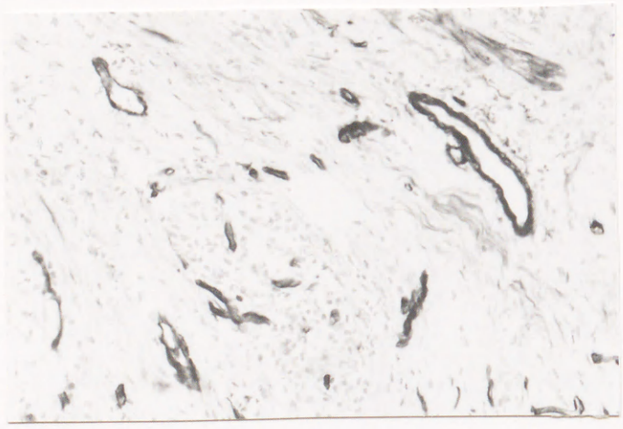


Fig 4

a



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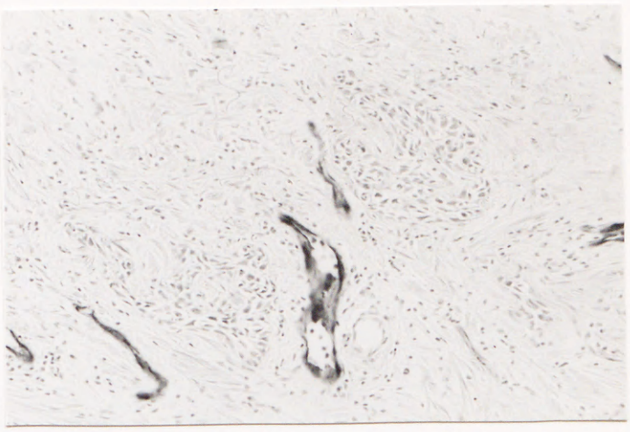

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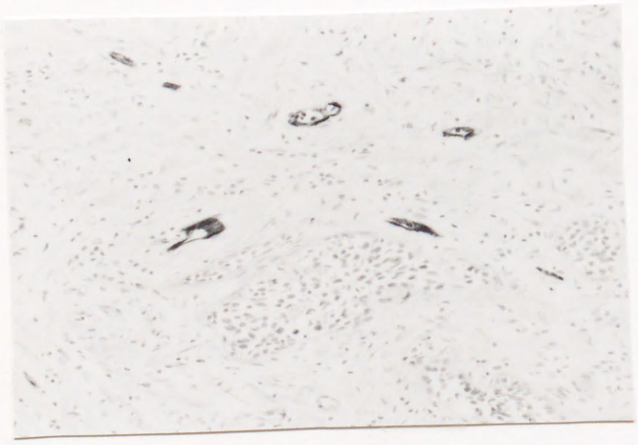
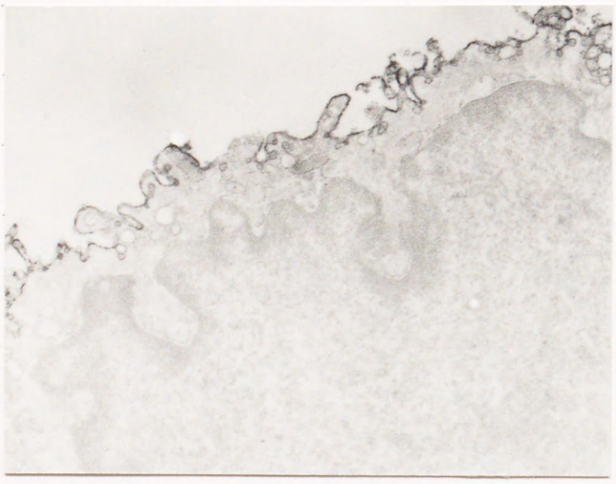


Fig 5

a



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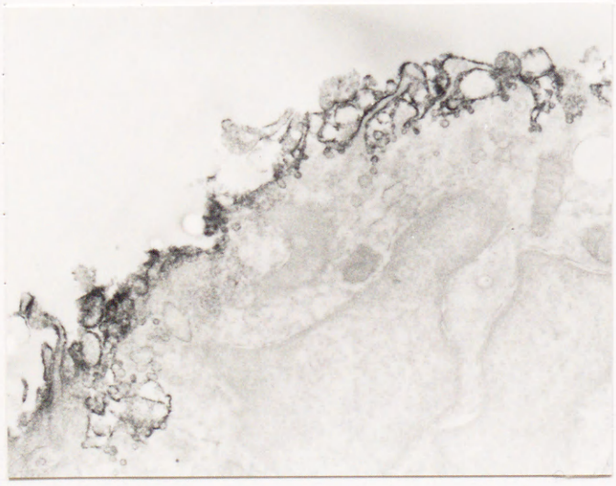


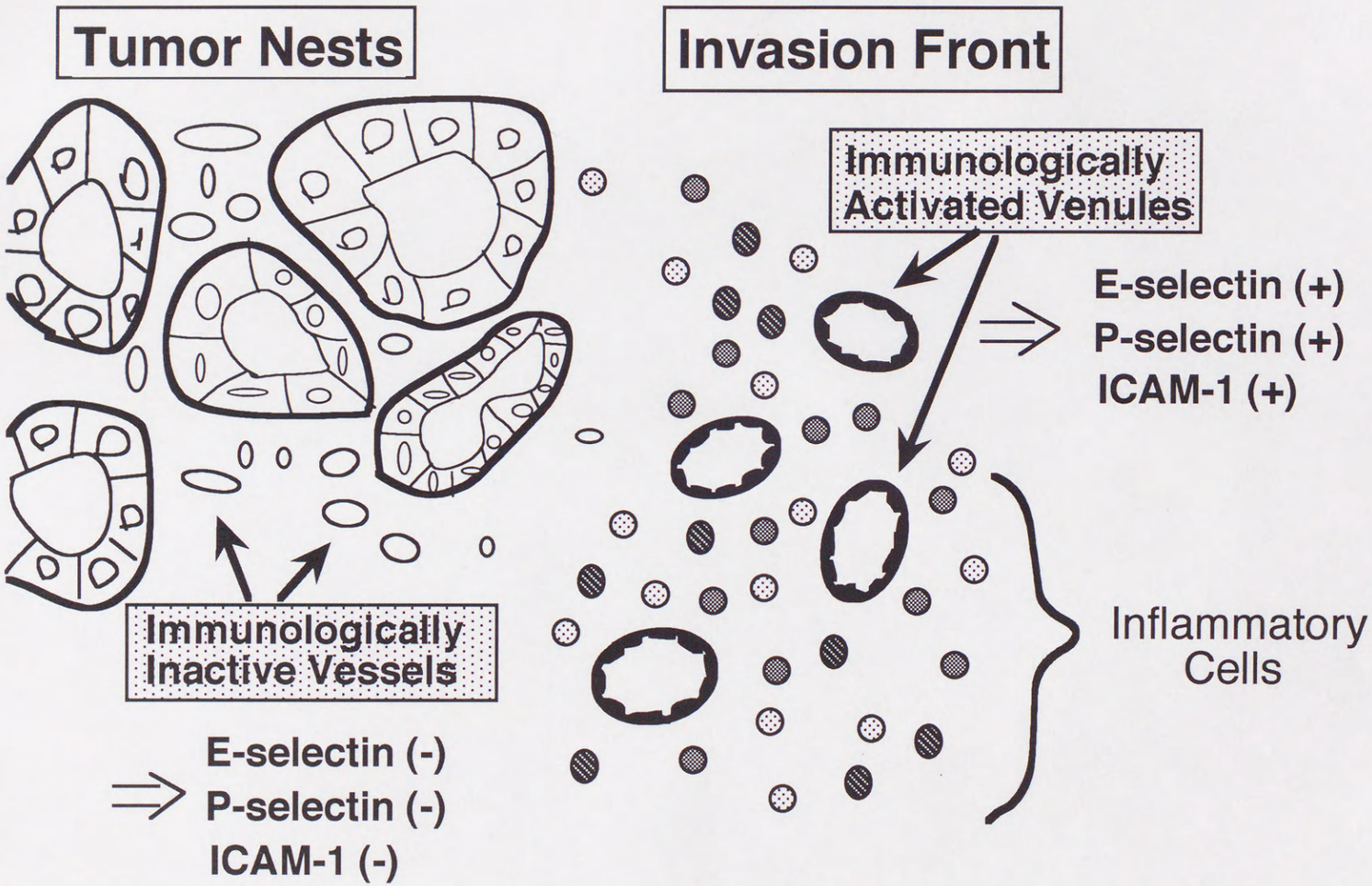

Tumor Nests
Invasion Front
Immunologically Activated Venules
E-selectin (+)
P-selectin (+)
ICAM-1 (+)
Immunologically Inactive Vessels
E-selectin (-)
P-selectin (-)
ICAM-1 (-)
Inflammatory Cells

Fig 6

Fig 7

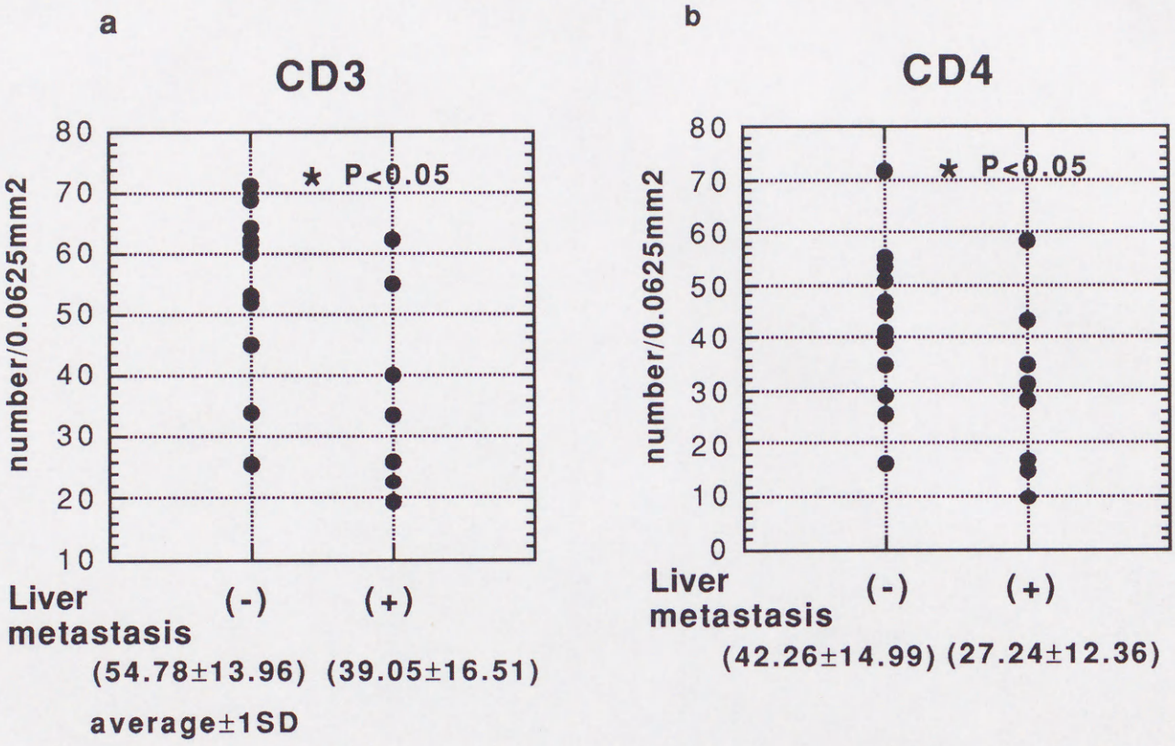
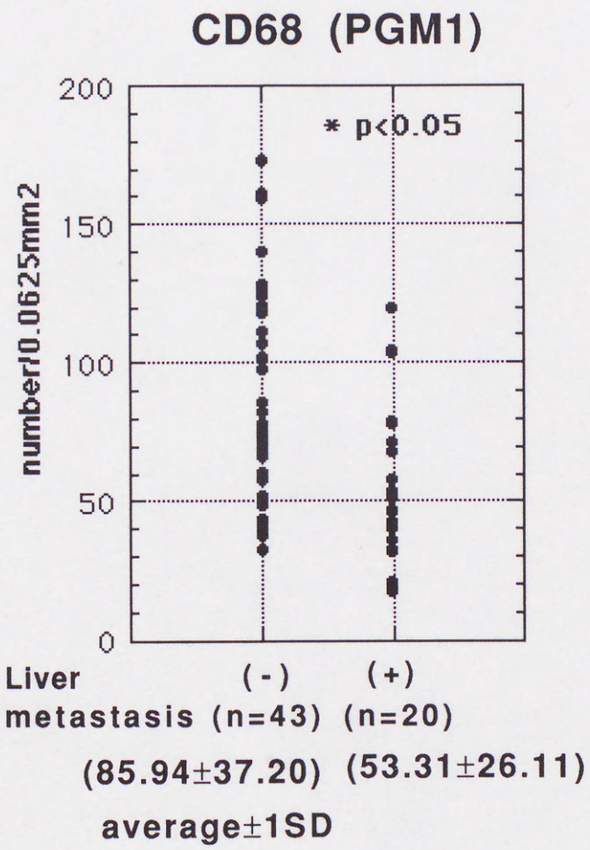
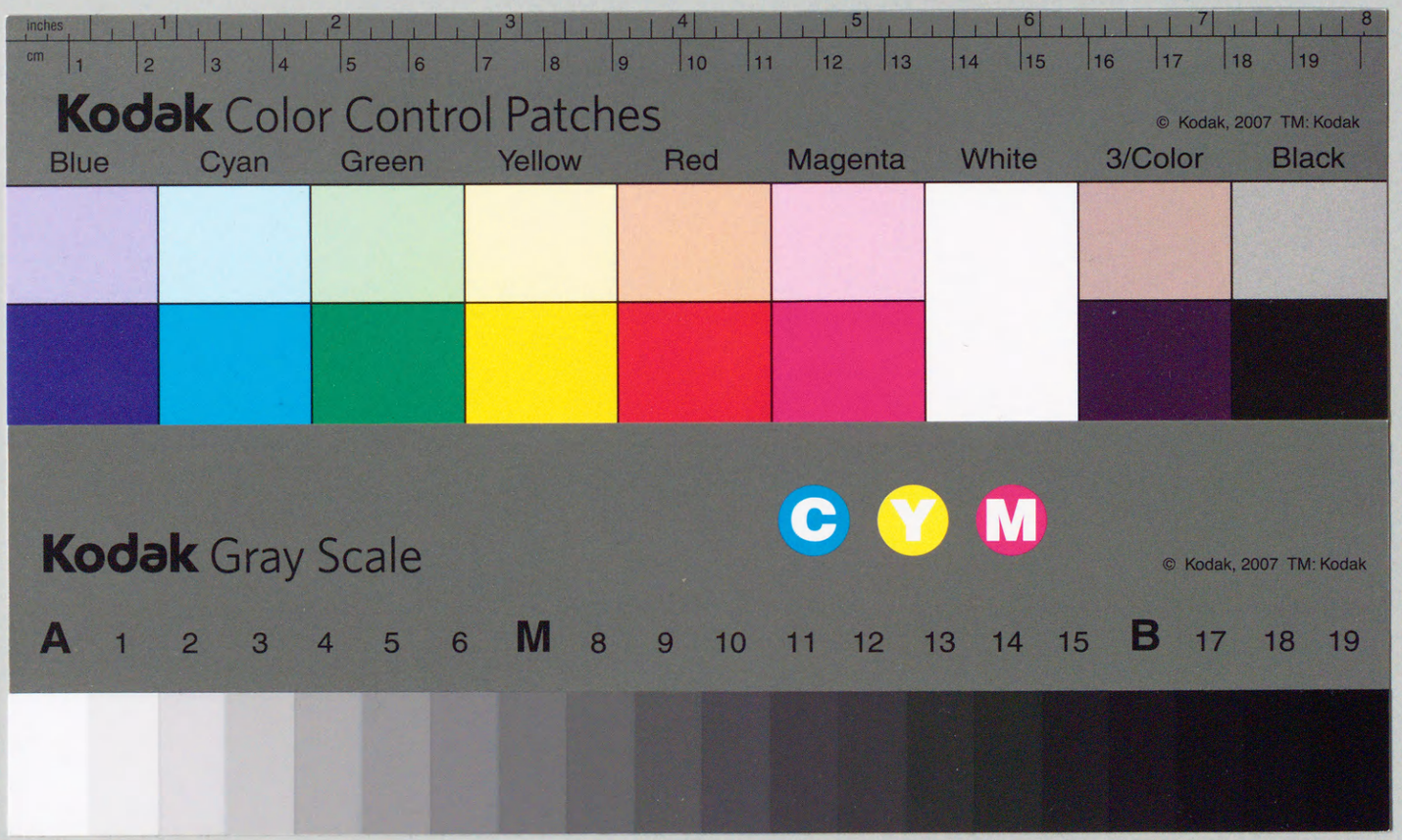

a
CD3
number/0.0625mm2
80
70
60
50
40
30
20
10
* P<0.05
Liver metastasis
(-)
(+)
(54.78±13.96)
(39.05±16.51)
average±1SD
b
CD4
number/0.0625mm2
80
70
60
50
40
30
20
10
0
* P<0.05
Liver metastasis
(-)
(+)
(42.26±14.99)
(27.24±12.36)

Fig 8


CD68 (PGM1)
* p<0.05
number/0.0625mm2
200
150
100
50
0
Liver metastasis
(-) (n=43)
(+) (n=20)
(85.94±37.20)
(53.31±26.11)
average±1SD


inches
cm
Kodak Color Control Patches
© Kodak, 2007 TM: Kodak
Blue
Cyan
Green
Yellow
Red
Magenta
White
3/Color
Black
Kodak Gray Scale
C
Y
M
© Kodak, 2007 TM: Kodak
A 1 2 3 4 5 6 M 8 9 10 11 12 13 14 15 B 17 18 19